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AWARD NUMBER:
W81XWH-10-1-0344

TITLE:
Metabolic regulation of ovarian cancer cell death

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REPORT DATE:

July 2013

TYPE OF REPORT:
Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release, distribution unlimited

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# REPORT DOCUMENTATION PAGE

*Form Approved*
*OMB No. 0704-0188*



| 1. REPORT DATE *(DD-MM-YYYY)* | 2. REPORT TYPE | 3. DATES COVERED *(From - To)* |
|---|---|---|
| July 2013 | Final | 1 JULY 2010 - 30 JUNE 2013 |

**4. TITLE AND SUBTITLE**

Metabolic Regulation of Ovarian Cancer cell death

**5a. CONTRACT NUMBER**

**5b. GRANT NUMBER**
W81XWH-10-1-0344

**5c. PROGRAM ELEMENT NUMBER**

**6. AUTHOR(S)**

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**5d. PROJECT NUMBER**

**5e. TASK NUMBER**

**5f. WORK UNIT NUMBER**

**7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)**

**AND ADDRESS(ES)**
Duke University School of Medicine

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**8. PERFORMING ORGANIZATION REPORT NUMBER**

**9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)**
**US Army Medical Research**
**Materiel Command, Fort Detrick, Maryland 21702-5012**

**10. SPONSOR/MONITOR'S ACRONYM(S)**

**11. SPONSOR/MONITOR'S REPORT NUMBER(S)**

**12. DISTRIBUTION / AVAILABILITY STATEMENT**
Approved for public release, distribution unlimited

**13. SUPPLEMENTARY NOTES**

**14. ABSTRACT**
Following treatment with chemotherapeutic agents, responsive ovarian cancer cells undergo apoptotic cell death. Several groups have shown that the apoptotic protease, caspase 2 (C2), is an essential activator of cell death in ovarian cancer cells treated with cisplatin and we have found, by knock-down of C2 in ovarian cancer cells, that C2 is also required for responsiveness to microtubule perturbing agents such as paclitaxel. Work from our laboratory has demonstrated that C2 is normally controlled by the metabolic status of the cell in that high levels of flux through the pentose phosphate pathway (PPP) prevents activation of C2 . This inhibition is exerted through phosphorylation of C2 at a specific residue that is catalyzed by calcium-calmodulin-dependent kinase II (CaMKII). CaMKII is activated by the product of the PPP, NADPH, through its ability to support fatty acid synthesis. The precise mechanism by which fatty acid synthesis activates CaMKII is not yet known.
Because ovarian cancers exhibit increased glucose uptake and increased fatty acid synthesis**,** we **hypothesized** that susceptibility of ovarian cancers to front-line chemotherapeutic agents, reflect, at least in part, the metabolic status of the cells and, consequently, the phosphorylation state of caspase 2. We have found that inhibition of PPP operation and interference with fatty acid synthesis sensitizes ovarian cancer cells to a range of chemotherapeutic agents that depend upon C2 for cell death.

**15. SUBJECT TERMS**
OVARIAN CANCER, FATTY ACID SYNTHESIS, CHEMOTHERAPY, CASPASE 2, G6PD

| 16. SECURITY CLASSIFICATION OF: | | | 17. LIMITATION OF ABSTRACT | 18. NUMBER OF PAGES | 19a. NAME OF RESPONSIBLE PERSON |
|---|---|---|---|---|---|
| a. REPORT U | b. ABSTRACT U | c. THIS PAGE U | UU | 9 | 19b. TELEPHONE NUMBER *(include area code)* |

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**Introduction:** This proposal stemmed from our observations in model systems demonstrating that the apoptotic protease, caspase 2, is critical for cell death in response to various chemotherapeutic agents. In addition, we had found that caspase 2 activity could be modulated by altering the metabolic status of cells[1]. We proposed to determine whether ovarian cancer cells were, indeed, dependent on caspase 2 for cell death and, in addition, whether metabolic manipulation could enhance ovarian cancer cell death. If so, we wished to determine whether this enhanced cell death could be correlated with changes in caspase 2 phosphorylation or oligomerization status, which are indicative of caspase 2 activation. Finally, we proposed to determine whether the phosphorylation status of caspase 2 or the metabolomic profile of ovarian cancer cells could predict responsiveness of ovarian tumors to chemotherapy.

Keywords: OVARIAN CANCER, FATTY ACID SYNTHESIS, CHEMOTHERAPY, CASPASE 2, G6PD

**Overall Project summary:**

This project has been productive and we are continuing to pursue the original SOW (reproduced below) going forward.

Statement of Work:

Task 1: To analyze caspase 2 - dependence of apoptosis in ovarian cancer cell lines (months 1-10):

Test a panel of ovarian cancer cell lines with multiple chemotherapeutic agents following ablation of caspase 2 using siRNA. (months 1-3)

Deconvolute siRNA pools and construct siRNA - resistant caspase 2 mutants (months 4-6).

Assay of phosphomimetic and non - phosphorylatable caspase 2 variants in ovarian cancer cells (months 6-10)

Task 2: To assess the role of metabolic intermediates in controlling ovarian cancer cell death (months 10-24):

Determine the responsiveness of ovarian cancer cell lines to C75 and C93 (months 10-13)

Determine the phosphorylation and oligomerization status of caspase 2 in cell lines treated with agents that alter

| | IC 20% | IC 50% | IC 80% | Lower limit % |
|---|---|---|---|---|
| **OVCAR3** | | | | |
| Cisplatin | 3μM | 6μM | -- | 35% |
| Gemcitabine | -- | -- | -- | 90% |
| Paclitaxel | <1nM | 1nM | -- | 40% |
| **OVCAR4** | | | | |
| Cisplatin | 2μM | 10μM | 75μM | 10% |
| Gemcitabine | <10nM | 250nM | -- | 45% |
| Paclitaxel | 50nM | -- | -- | 70% |
| **ES2** | | | | |
| Cisplatin | 2.5μM | 10μM | 75μM | 10% |
| Gemcitabine | <10nM | 10nM | -- | 45% |
| Paclitaxel | <8nM | 100nM | -- | 40% |
| **A2780S** | | | | |
| Cisplatin | 300nM | 1μM | 10μM | 10% |
| Gemcitabine | <8nM | <8nM | 1μM | 15% |
| Paclitaxel | 1nM | 5nM | -- | 30% |
| **A2780R** | | | | |
| Cisplatin | 4μM | 30μM | -- | 25% |
| Gemcitabine | <10nM | 100nM | -- | 40% |
| Paclitaxel | 1nM | 3nM | 100nM | 20% |

**Table 1.** The lower limit % is the percentage of cells that are still alive even at maximum dose of the drug

metabolism (months 13-19)

Perform proof-of-concept experiments to determine if CaMKII inhibitors would be viable chemotherapeutic agents in ovarian cancer and assess their effects on caspase 2 in ovarian cancer cells (months 19-24).

Task 3: To assess the phosphorylation status of caspase 2 in primary ovarian tumors and to develop metabolomic signatures for ovarian cancer cells/tumors (months 25-36):

Analyze caspase 2 phosphorylation in tumors of different histological sub-types and grade (months 25-28)

Perform metabolomic analysis of tumor samples (months 28-35)

Analyze metabolomic data and construct metabolomic signatures for ovarian tumors (months 35-36)

To assess the ability of metabolic inhibitors to enhance apoptosis in ovarian cancer cells, we first undertook a careful titration of standard chemotherapeutic agents in an array of ovarian cancer cells. Based on the data shown in **Table I**, we are confident that we can find appropriate doses to see any enhancement of cell death through metabolic inhibition.

As detailed at the end of our first year of funding, we were able to demonstrate caspase 2-dependence of apoptosis in ovarian cancer cells in response to a number of chemotherapeutic agents (e.g., **Fig. 1**), as proposed in Task I.

Throughout the course of this proposal, we had the greatest success with Task 2 (which is also the most immediately relevant to discovering new therapeutic strategies). As described in last year's report, we found that fatty acid synthase inhibitors very effectively killed ovarian cancer cells (**Fig. 2**) and we traced this activity to the induction of a protein known as REDD1 (**Fig. 3**). In this year, we have found that the action of REDD1 is due to its ability to inhibit the mTOR pathway and that mTOR inhibition could substitute for REDD1 induction to allow cell death in ovarian cancer cells that were otherwise resistant to fatty acid synthase inhibitors **Fig. 4**). We were slightly delayed in submitting this work for publication as the first author had to finish his PhD thesis and graduate, but we anticipate submission in the next few months.

While working on metabolic control of caspase 2, as described in Task 2, we found that inhibition of the pentose phosphate pathway (PPP) using the steroid DHEA (also an allosteric inhibitor of the rate limiting enzyme of the PPP, G6PD) could enhance caspase 2-dependent death of ovarian cancer cells in response to a variety of chemotherapeutic agents (**Fig. 5**). These observations strongly suggested that G6PD inhibition might synergize with chemotherapeutic agents to yield an enhanced therapeutic response in ovarian cancer. DHEA treatment induced dephosphorylation of human caspase 2 at S164 (a site we had previously shown could suppress caspase 2 activity). Importantly, this was effectively antagonized by treatment with a cell permeable variant of malate (Dimethyl-L-malate), which with malic enzyme, promotes production of NADPH outside of the PPP (**Fig.6**). To obtain direct evidence that manipulating NADPH levels modulates caspase 2 activation, we employed a fluorescent indicator of caspase 2 activation originally reported by Douglas Green's laboratory[2]. DHEA treatment (to decrease

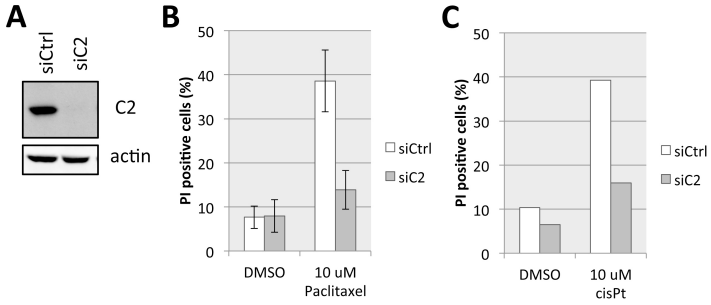


**Fig. 1.** B, C. DOV-13 ovarian cancer cells were treated with siRNA directed against caspase 2. They were then treated with the indicated chemotherapeutic agents (paclitaxel or cisplatin) and cell death was monitored by PI staining and flow cytometry. A. A representative immunoblot showing caspase 2 knockdown is also shown. Similar results were obtained in several ovarian cancer cell lines.

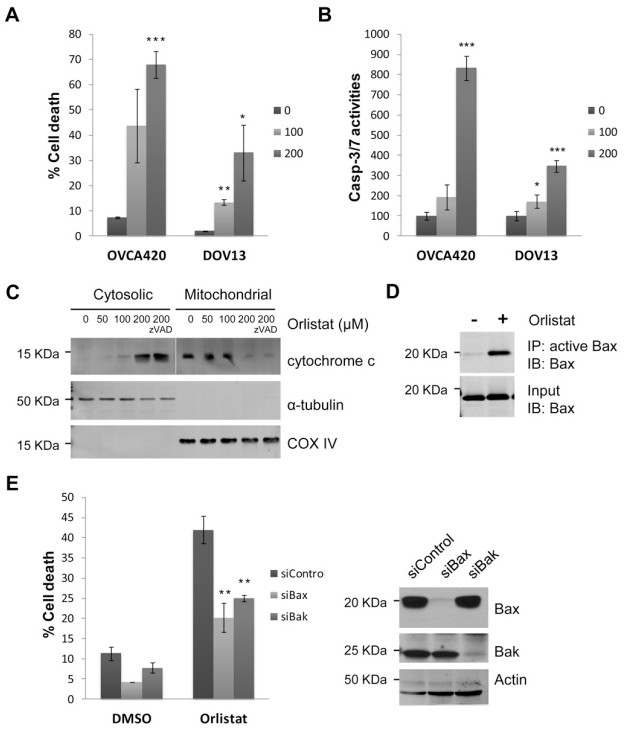


**Fig. 2.** Orlistat induces apoptosis in ovarian cancer cells through the intrinsic pathway. **A**, Ovarian cancer cells was treated with indicated dose of orlistat (0, 100, or 200 μM). The PI-positive population was monitored 24h post treatment by flow cytometry. **B**, Orlistat induced dose-dependent activation of effector caspases. Cells were treated as shown in A and then lysed for a caspase assay. **C**, Inhibition of FASN by orlistat triggered cytochorme c release from mitochondria in OVCA420 cells. COX IV and α-tubulin were used as loading controls and markers for mitochondrial and cytosolic fractions. **D**, Orlistat-induced Bax activation in OVCA420 cells was examined using a monoclonal antibody (6A7) raised against the active form of Bax. **E**, Downregulation of either Bax or Bak, the primary MOMP regulators, by RNAi significantly rescued orlistat-induced cell death in OVCA420 cells. Lysate from transfected cells was resolved by immunoblotting for the efficacy of different siRNAs. (*P<0.05, **P<0.01, ***P<0.001)

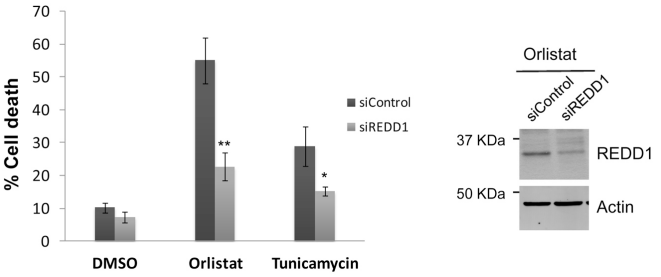


**Fig. 3.** REDD1 RNAi protects cells from orlistat- and tunicamycin-induced death. Transfected OVCA420 cells were treated with DMSO, 200 μM orlistat or 10 μg/ml tunicamycin for 24 h. PI-positive cells were analyzed by flow cytometry. The efficacy of REDD1 siRNA was examined by immunoblotting. (*P<0.05, **P<0.01)

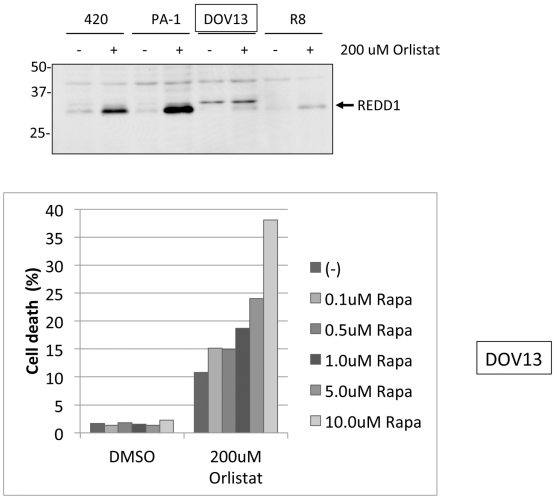


**Fig. 4.** A. Various ovarian cancer cell lines were treated with orlistat and lysates were immunblotted for REDD1. B. DOV 13 cells which fail to induce REDD1 are relatively resistant to orlistat, but treatment with the mTOR inhibitor, rapamycin, restores sensitivity to orlistat-induced death (as measured by PI staining and flow-cytometry).

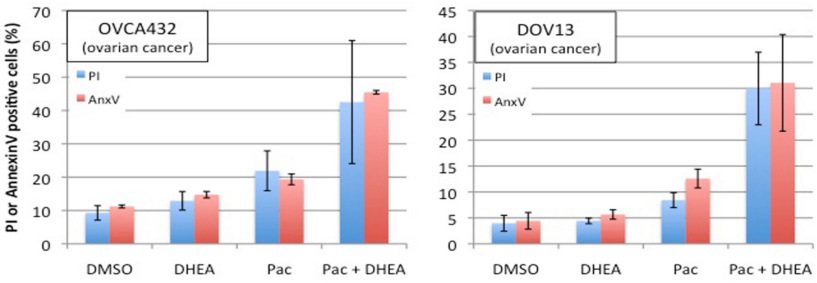


**Fig. 5.** Cells were treated with DMSO, DHEA, chemotherapeutic agent (paclitaxel or etoposide as labeled in panels) or chemotherapeutic plus DHEA and monitored by PI staining or annexin V staining (as indicated) and flow cytometry.

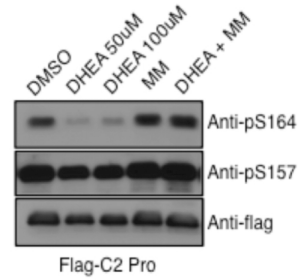


**Fig. 6.** Cells were transfected with the prodomain from caspase 2 and treated with either DHEA at the indicated concentrations or Dimethyl-L-malate (indicated as MM). Cell lysates were resolved by SDS-PAGE and immunoblotted with anti-phospho S164 antibody.

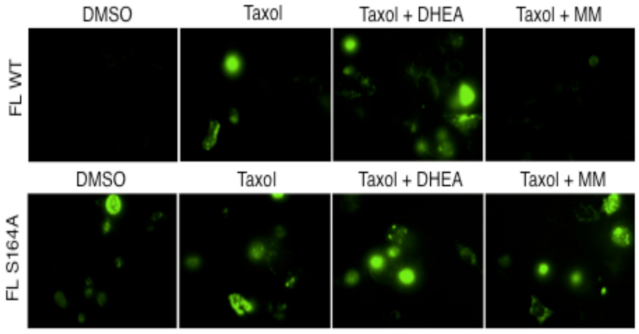


**Fig. 7.** Cells were co-transfected with WT or S164A mutant caspase 2 prodomain constructs fused to either the N or C terminus of fluorescent venus protein. Cells were treated with either DMSO, taxol, taxol plus DHEA or taxol plus dimethyl-L-malate and monitored by fluorescent microscopy.

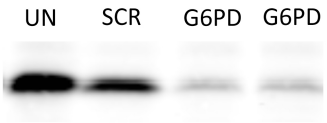


**Fig 8**. ES2 cells were infected with retroviral vectors carrying either no shRNA, scrambled shRNA, or two different shRNAs directed against G6PD. Cells were lysed and immunoblotted with anti-G6PD antibody.

NADPH) induced this fluorescence (and enhanced taxol-induce fluorescence), whereas Dimethyl-L-malate entirely suppressed caspase 2 activation (**Fig. 7**). Identical experiments using an S164A mutant (unphosphorylatable) human caspase 2 resulted in constitutive fluorescence regardless of metabolic status, consistent with this being the site of metabolic regulation. (**Fig. 7)**. While DHEA is not a suitable drug (as a precursor of reproductive steroids, it might adversely affect the course of ovarian cancer), a number of more potent/specific inhibitors of G6PD have been recently identified. These might either serve as therapeutics or provide lead compounds for therapeutic development. Accordingly, we have been extremely fortunate to enlist two collaborators with some of these novel G6PD inhibitors [3, 4] and we are poised to test these in ovarian cancer cells. As a proof of principle, we have now also stably knocked down G6PD in ovarian cancer cells (e.g., **Fig. 8** with ES 2 cells) and we will determine whether G6PD knockdown can synergize with chemotherapeutic agents to kill these cells.

We have not yet completed experiments described in Task 3.

**Key research accomplishments:**

- Identification of the intrinsic pathway as mediator of orlistat-induced death of ovarian cancers

- Demonstration that caspase-2 is activated by orlistat and conventional chemotherapeutic agents, leading to activation of Bak/Bax

- Demonstration that the 14-3-3 binding ability of REDD1 is required for its ability to mediate caspase 2 activation and cell death in response to orlistat

- Demonstration that REDD1 is required for orlistat-induced death in ovarian cancer cells

- Demonstration that the key activity of REDD1 in killing ovarian cancer cells is to inhibit the mTOR kinase

- Demonstration that DHEA can enhance killing of ovarian cancer cells by chemotherapeutic agents

- Successful knock-down of G6PD in ovarian cancer cells

**Conclusion:**

We have demonstrated that inhibition of fatty acid synthesis and inhibition of G6PD in ovarian cancer cells engages caspase 2 to promote cell death. In the case of fatty acid synthesis inhibition, the activation of caspase 2 depends upon the ability to promote activation of REDD1. We will follow up on the proof of principle experiments with DHEA and G6PD knock-down by using novel inhibitors of G6PD to determine whether these might serve as novel chemotherapeutic agents for ovarian cancer.

**Publications, Abstracts and Presentations:**

Keystone Symposium on Tumor Metabolism, Feb 24-March 1, 2013
*Metabolic Control of Caspase 2-Mediated Cell Death*

Gordon Research Conference on Cell Proliferation, June 23-28, 2013
*Killing cancer cells through inhibition of fatty acid synthesis*

**Reportable outcomes:**

Manuscript nearing submission demonstrating involvement of REDD1 in ovarian cancer death after inhibition of fatty acid synthesis inhibition.

**Other Achievements:**

Creation of ovarian cancer cells in which G6PD has been stably knocked down.

**Literature cited:**

1. Nutt, L.K., S.S. Margolis, M. Jensen, C.E. Herman, W.G. Dunphy, J.C. Rathmell and S. Kornbluth, *Metabolic regulation of oocyte cell death through the CaMKII-mediated phosphorylation of caspase-2.* Cell, 2005. **123**(1): p. 89-103.

2. Bouchier-Hayes, L., A. Oberst, G.P. McStay, S. Connell, S.W. Tait, C.P. Dillon, J.M. Flanagan, H.M. Beere and D.R. Green, *Characterization of cytoplasmic caspase-2 activation by induced proximity.* Molecular cell, 2009. **35**(6): p. 830-40.
3. Preuss, J., A.D. Richardson, A. Pinkerton, M. Hedrick, E. Sergienko, S. Rahlfs, K. Becker and L. Bode, *Identification and characterization of novel human glucose-6-phosphate dehydrogenase inhibitors.* Journal of biomolecular screening, 2013. **18**(3): p. 286-97.
4. Hamilton, N.M., M. Dawson, E.E. Fairweather, N.S. Hamilton, J.R. Hitchin, D.I. James, S.D. Jones, A.M. Jordan, A.J. Lyons, H.F. Small, G.J. Thomson, I.D. Waddell and D.J. Ogilvie, *Novel steroid inhibitors of glucose 6-phosphate dehydrogenase.* Journal of medicinal chemistry, 2012. **55**(9): p. 4431-45.